Comparison of immune parameters in cows with normal and prolonged involution time of uterus

M. Levkut, J. Pistl, V. Revajová, J. Choma, M. Levkutová, V. Dávid

University of Veterinary Medicine, Košice, Slovak Republic

ABSTRACT: Indices of cellular immunity in postpartum Holstein cows with the normal ($n = 9$) and prolonged ($n = 4$) uterine involution time were evaluated. Peripheral white blood cells were isolated by lysis from postpartum animals. An indirect immunofluorescence method for staining and flow cytometric analysis was employed to determine the cell subpopulation of lymphocytes. The function assays were also used to examine the activity of lymphocytes and phagocytes. A significant decrease in the lymphocyte absolute number, and subpopulation of T (CD2+, CD4+, CD8+), and B (IgM+) cells in dams with postparturient complications and prolonged uterine involution time was observed. The quantitative changes of immune cells were accompanied by a significant decline of phagocyte functional activity in an iodo-nitro-tetrazolium reductase test and polyclonal lymphocyte activation to phytohemagglutinin in a leukocyte migration-inhibition assay. In conclusion, a significant decrease in the lymphocyte absolute number and subpopulation of T (CD2+, CD4+, CD8+), and B (IgM+) cells was observed and the host defense role of phagocytes and lymphocytes was impaired in cows with prolonged uterine involution, which can increase their susceptibility to infections.

Keywords: dairy cows; periparturient period; phagocytes; lymphocytes; cell-mediated immune functions; immunosuppression; flow cytometry

During the periparturient period, animals undergo pronounced physiological changes that might cause suppression of the host defense mechanisms including both the cellular and humoral response of the immune system and an increase in susceptibility to uterine and mammary gland infection. The involutionary processes of genital organs are a complex involving contraction, loss of tissue connected with the high incidence of bacterial contamination of the uterus, tissue repair (epithelization) and resumption of ovarian activity. Factors contributing to these changes have not been fully elucidated although major shifts in the plasma concentration of steroids and arachidonic acid metabolites have been proposed to play a role (Cai *et al.*, 1994).

Gravidity influences the immune system and the following clinical signs of prospective dam diseases. The decrease in some immunological parameters in pregnant cows was published, mainly in the number of peripheral blood lymphocytes, T (CD4, CD8), B, and NK lymphocytes (Gleicher and Siegel, 1983; Iwatani *et al.*, 1988). Sato (1998) recorded the decreased values of T lymphocyte proliferative activity on mitogen activation, and cytotoxic activity of K and NK cells. Moreover, the decline of IgG, IgA, and IgM in the blood serum was found (Amino *et al.*, 1978). These changes in the immunological parameters were also detected during the postpartum period in cows suffering from mastitis and metritis (Sato *et al.*, 1995; Sato, 1998).

The aim of this study was to select dams suffering from postpartum complications under the conditions of conventional cattle breeding with optimal gravidity course and to compare their cellular immunity parameters with those of healthy cows.

MATERIAL AND METHODS

Animals

An experiment was carried out on 13 cows of the Holstein breed. Animals were aged 3 years and had optimal gravidity course during 260 days.

Examination per rectum was done 3 times weekly to evaluate the process of uterine involution. At each examination we recorded cranial delineation of the uterine horns, uterus localization in the abdominal or pelvic cavity, diameters of the uterine body and horns according to the method by Studer and Morrow (1980).

The cows were divided into two groups according to the uterine involution time – group with normal uterine involution ($n = 9$) and group with prolonged uterine involution ($n = 4$).

Blood sample collection

Blood samples for flow cytometry and function leukocyte assays were collected from the *vena jugularis* into 1.5% EDTA (0.1 ml/1 ml of blood; Lachema, Brno, Czech Republic) on days 1, 7, 14, and 21 after parturition.

Counting of total and differential leukocytes

Blood was transferred into Türk's solution (at a dilution 199 : 1) and the cells were counted in Bürker's chamber. Differential cell counts were made on blood smears after Haemacolor commercial kit (Merck, Germany) staining, by counting 100 cells per slide.

Computation of absolute leukocyte counts. Absolute leukocyte counts were computed as follows:

WBC count × % of differential types of leukocyte

Flow cytometry

Indirect immunofluorescence method and whole blood were used to measure relative percentage of lymphocyte subpopulations. Mouse anti-bovine primary monoclonal antibodies against CD2, CD4, CD8 (T lymphocytes) and IgM (B lymphocytes) were used (International Laboratory for Research on Animal Diseases – ILRAD, Kenya) and are summarized in Table 1. FITC-conjugated F(ab′)$_2$ fragment of goat anti-mouse immunoglobulins was used as a secondary antibody (Dako, Denmark).

Procedure for flow cytometry (FACS). The whole blood hemolysis was done using a lysing solution. One hundred μl was lysed with 1.4 ml lysing solution (NH$_4$Cl 8.26 g, NaHCO$_3$ 1.09 g, NaEDTA 0.037 g was solved in 100 ml distilled water and then water was added to 1 l). White blood cells were washed 3× with PBS (phosphate buffered saline – Sigma, Germany), and incubated with diluted specific MoAbs at 4°C for 30 minutes in the dark. For controls non-specific isotype control was used. After incubation the samples were washed 2× with PBS and incubation with secondary antibody followed as described above. The samples were washed 2× with PBS and fixed for measuring in 1% paraformaldehyde (diluted in PBS; Sigma, Germany).

FACScan and Cell Quest programme (Becton Dickinson, Germany) were used for measuring. The white blood cells were measured, mononuclear cells were gated by forward and side light scatter. The data on 10 000 lymphocytes were collected, and the relative percentages obtained from the cytometry analysis were used to compute the absolute counts of the subpopulation of lymphocytes as follows:

absolute lymphocyte counts × relative percentage of positive cell subpopulations

Function assays for evaluation of phagocyte and lymphocyte activity

Leukocytes were isolated from the peripheral blood by the method of osmotic shock of erythrocytes by distilled water according to Karlson and Kaneko (1973).

Iodo-nitro-tetrazolium reductase test (INT). Quantitative evaluation of the tetrazolium-reductase activity of phagocytes was carried out according to the method of Lokaj and Obůrková (1986) for the evaluation of the metabolic activity (MA) of phagocytes during phagocytosis. The leukocyte suspension (1.10^7/ml) was divided in two parts and incubated at 37°C for 45 minutes. One portion was incubated with 1% starch suspension (Amylum oryzae) in phosphate buffer saline (PBS) and the other without starch. All cellular suspensions contained INT (3/4-iodophenyl-2-/4-nitrophenyl/-5-phenyl/-tetrazolium chloride; Lachema, Brno, Czech Republic). After the incubation and lysis of cells with acetone, the formasan content was determined with spectrophotometer at 485 mm. The results are described in the form of an index of metabolic activity (IMA) based on the ratio of the

Table 1. Primary monoclonal antibodies (MoAbs) used for indirect immunofluorescent staining of lymphocyte subpopulations

Specificity	MoAbs	Isotype	Dilution
CD2	IL-A42	IgG2a	1 : 1 000
CD4	IL-A12	IgG2a	1 : 2 000
CD8	IL-A51	IgG1	1 : 5 000
IgM (μ chain)	IL-A30	IgG1	1 : 2 500

mean optical density of leukocyte suspensions with starch and leukocyte suspensions without starch.

Leukocyte migration-inhibition assay (LMIA). LMIA was used to analyze the reaction capacity of lymphocytes to mitogenic activation and was carried out according to Bendixen *et al.* (1976). The leukocyte suspensions were tested at the concentration of 2.10^8/ml under agarose with 5 μg/ml mitogen (phytohemagglutinin, PHA, Sigma, Germany) and without mitogen. The plastic dishes contained 5 ml of 1% agarose as a culture medium (RPMI 1640, 10% foetal calf serum, 100 μg/ml of streptomycin and 100 IU/ml penicillin). After pre-incubation (37°C, 1 h, 5% CO_2) the cell suspensions were transferred to wells in the agarose medium and incubated under the same conditions for 20 hours. Five wells with mitogen and five without it were used for all samples. PHA-activated T lymphocytes produce a higher level of lymphokins including the migration inhibition factor (MIF) which causes the inhibition of leukocyte migration (monocytes, polymorphonuclears). The intensity of migration inhibition of these leukocytes depends on MIF amount produced by activated lymphocytes. The areas of leukocyte migration were measured after magnification with planimeter and the results were expressed as migration index (MI), represented as the ratio of mean areas of leukocyte migration with PHA to the area of leukocyte migration without PHA. MI < 0.9 indicates a positive reaction – activation of T lymphocytes with PHA and more intensive activation of lymphocytes by mitogen occurs at a lower value of MI.

Statistical evaluation

For statistical analysis the unpaired *T*-tailed test was used.

RESULTS

In cows with prolonged uterine involution time a significant decrease in the absolute number of lymphocytes (Figure 1), and CD2+, CD4+, and CD8+ T cells on days 7, 14, and 21 after parturition (Figures 2, 3, 4) was detected. Significant depletion of IgM expressed B cells (Figure 5) from the first day after parturition to the end of the study was observed in this group compared with the healthy group of cows with normal uterine involution time. No significant differences between the two groups were recorded in the ratio of CD4/CD8 T cells nor in the total number of leukocytes, and absolute number of neutrophils, eosinophils and monocytes.

The animals with prolonged uterine involution showed a significant decrease in the phagocyte metabolic activity ($P < 0.01$) compared with normal involution cows (Figure 6). A significant increase in the leukocyte migration index was observed in dams with prolonged uterine involution, which refers to a decline of the polyclonal lymphocyte activation to mitogen – phytohemagglutinin ($P < 0.1$; Figure 7).
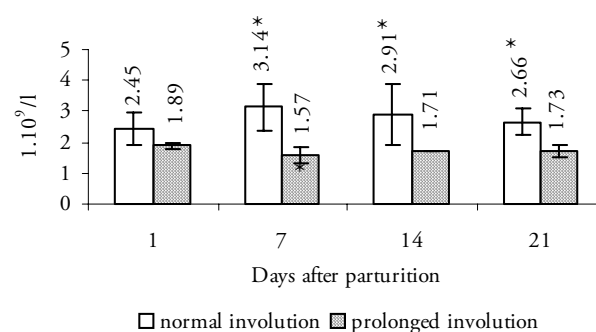

Figure 1. Absolute number of lymphocytes in the peripheral blood of cows (means ± SD; *$P < 0.01$)

Figure 2. Absolute number of CD2 positive T lymphocytes in the peripheral blood of cows (means ± SD; *P < 0.05, **P < 0.005)
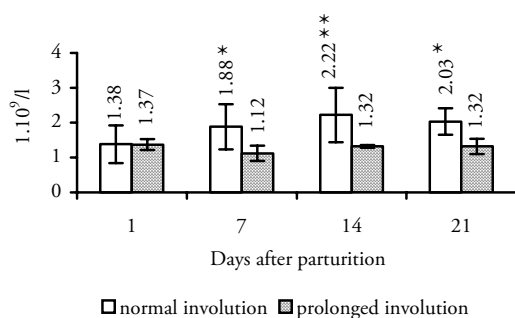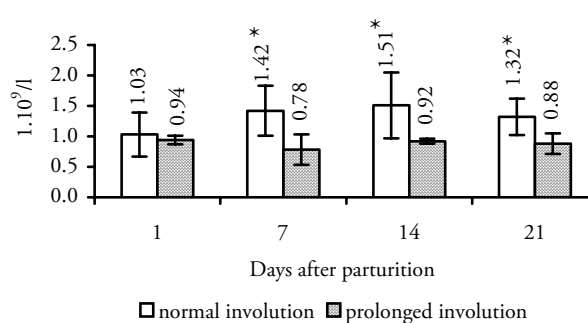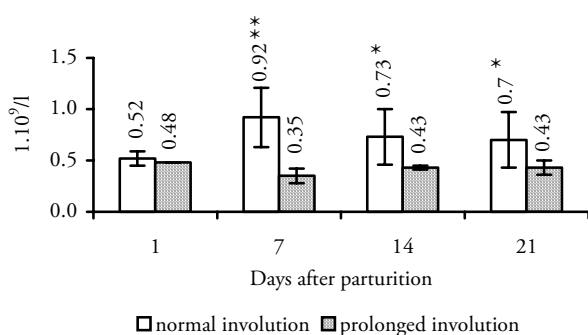

Figure 3. Absolute number of CD4 positive T lymphocytes in the peripheral blood of cows (means ± SD; *P < 0.05)

Figure 4. Absolute number of CD8 positive T lymphocytes in the peripheral blood of cows (means ± SD; *P < 0.05, **P < 0.001)
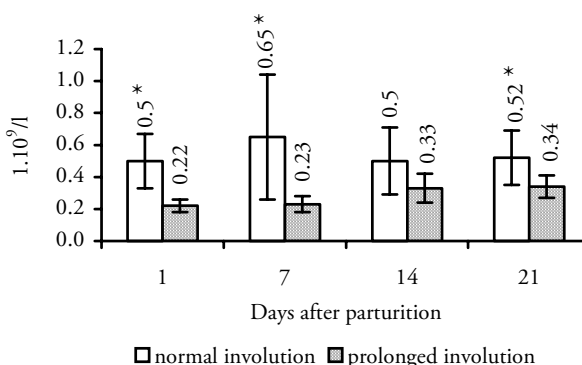

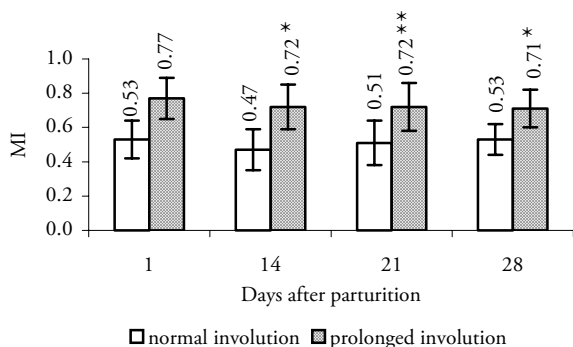Figure 5. Absolute number of IgM positive B lymphocytes in the peripheral blood of cows (means ± SD; *P < 0.05)

Figure 6. The values of leukocyte migration index after activation of lymphocytes with phytohemagglutinin (means ± SD; *P < 0.01, **P < 0.001)
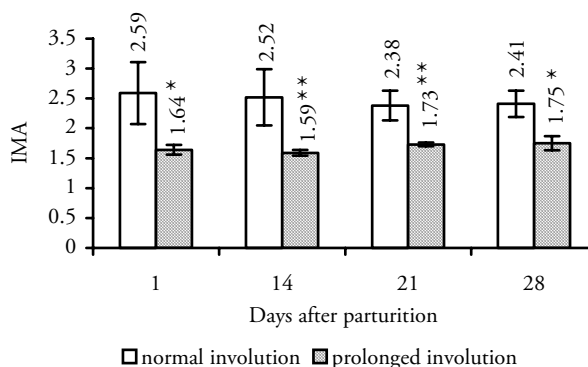

Figure 7. The values of metabolic activity index of phagocytes (means ± SD; *P < 0.01, **P < 0.001)

DISCUSSION

The postpartum uterus involution is basically an aseptic process, but parturition itself, disintegration of uterine caruncles and uterine lochia are very good conditions for uterus contamination and pathogen multiplication, notably in the case of immunosuppressive animals (Cai *et al.,* 1994). The impaired immune function in the periparturient period contributes to increased susceptibility of the cow to infectious diseases at the time of calving (Kimura *et al.,* 1999).

The effects of immunosuppression in the periparturient period by the occurrence of infectious diseases in cows were evaluated by Sato (1998). He determined that the mitogenic response of peripheral blood lymphocytes to mitogens decreased significantly in the periparturient period. Nitroblue tetrazolium reductase activity and expression of interleukin-2 mRNA by the peripheral blood neutrophils were also decreased in his study. Suppression of lymphocyte blastogenesis was correlated with a higher incidence of puerperal metritis of cows (Sato *et al.*, 1995). A decrease in the proliferative response of blood lymphocytes upon stimulation with three mitogens in the periparturient period was also described by Saad *et al.* (1989). Immunosuppression in periparturient cows is suggested to be related to the occurrence of infectious diseases such as mastitis and puerperal metritis (Mallard *et al.,* 1998). Several mechanisms for immunosuppression observed at the time of calving have been suggested. High circulating levels of hormones including estrogen, progesterone, and glucocorticoids near the onset of parturition can suppress important lymphocyte functions. Lower percentage of T lymphocytes and decreased CD2, CD4 and CD8 subsets of T lymphocytes were observed in the peripheral blood of healthy cows (Van Kampen and Mallard, 1997). In addition to changes in T lymphocyte numbers, there are changes in the function of certain subpopulations as well (Shafer-Weaver *et al.*, 1999).

Shafer-Weaver and Sordillo (1997) studied the immunoregulatory role of CD8+ cells during postpartum. Depletion of CD8+ lymphocytes from the whole cultures significantly decreased proliferation and cytotoxic ability of cells isolated from mid to late lactating animals. CD8+ T lymphocytes are mainly of a cytotoxic nature during mid to late lactation, but they are mainly suppressive in postpartum animals. In our experiment we observed a decrease in CD8+ T lymphocytes on 7 d after parturition, but along with this also a decline in CD2+, and CD4+ lymphocyte subpopulations. The participation of CD4+ T helper (T_H) lymphocytes is integral for an effective immune response. Shafer-Weaver *et al.* (1999) described that the reduced secretion of IL-2 and IFN-γ (products of the T_H-1 CD4+) during the postpartum period was correlated to an increase in the incidence of mastitis and was partially due to lower numbers and functions of lymphocytes at this time. They also reported that T_H-2 CD4+ T lymphocytes mainly secreted IL-4, IL-5 and IL-10 and promoted humoral immunity. The lower specific antibody titres were observed in dairy cows used in our experiment, immunized with ovoalbumin, suffering from postpartum complications in comparison with higher titres in cows without complications (Choma *et al.*, 2000). On the other hand, Nagahata *et al.* (1992) observed not only significant differences in B lymphocyte populations during the periparturient period, but also the numbers of plaque-forming cells that significantly decreased after parturition.

The presented results show a decrease in the cellular immunity parameters in dams with prolonged uterine involution. The decline was found in absolute number of lymphocytes, subpopulation of lymphocytes, functional activity of lymphocytes and phagocytes. The decrease in immunological parameters indicates immunosuppression in animals with postpartum complications, but it is not clear if immunosuppression was the primary cause of postpartum complications or only a component and/or a consequence of other processes taking place in the organism.

REFERENCES

Amino N., Tanisawa O., Miyai K., Tanska F., Hyashi C., Kawashima M. (1978): Changes of serum immunoglobulins IgG, IgA, IgM, and IgE during pregnancy. Obstet. Gynecol., *52*, 415–420.

Bendixen G., Bentzen K., Clausen J.E., Kjaer M., Saborg M. (1976): Inhibition of human leukocyte migration. In: Natvig J.B., Perlmann P., Wigzel H. (eds.): Lymphocytes. Isolation, fractionation and characterization. Scand. I. Immunol., suppl. 5, Universitetsforlaget, Nyegaard, Oslo, 244–267.

Cai T.Q., Weston P.G., Lund L.A., Brodie B., McKenna D.J., Wagner W.C. (1994): Association between neutrophil functions and periparturient disorders in cows. Am. J. Vet. Res., *55*, 934–943.

Choma J., Naď P., David V., Bírešová M. (2000): The observation of antibody production during puerperium in dairy cows. Folia Vet., *44*, 204–205.

Gleicher N., Siegel I. (1983): Pregnancy and Immune State. John Wiley and Sons, New York. 225–245.

Iwatani Y., Amino N., Tachi J., Kimura M., Ura I., Mori M. (1988): Changes of lymphocyte subsets in normal pregnant and postpartum women. Am. J. Reprod. Immunol. Microbiol., *18*, 52–55.

Karlson G.P., Kaneko J.P. (1973): Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med., *142*, 853–856.

Kimura K., Goff J.P., Kehrli M.E., Harp J.A. (1999): Phenotype analysis of peripheral blood mononuclear cells in periparturient dairy cows. J. Dairy Sci., *82*, 315–319.

Lokaj V., Obůrková P. (1986): Evaluation of tetrazolium-reductase activity of leukocytes (in Czech). 217–221. In: Procházková J., John C. (eds.): Selected Diagnostic Methods of Medical Immunology (in Czech). Avicenum, Zdravotnické nakladatelství, Praha.

Mallard B.A., Dekkers J.C., Ireland M.J., Leslie K.E., Sharif S., Vankampen C.L., Wagter L., Wilkie B.N. (1998): Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. J. Dairy Sci., *81*, 585–595.

Nagahata H., Ogawa A., Sanada Y., Noda H., Yamamoto S. (1992): Peripartum changes in antibody producing capability of lymphocytes from dairy cows. Vet. Q., *14*, 39–40.

Saad A.M., Concha C., Aström G. (1989): Alterations in neutrophil phagocytosis and lymphocyte blastogenesis in dairy cows around parturition. Zentralbl. Veterinarmed. B, *36*, 337–345.

Sato S. (1998): Immunosuppression in periparturient cows and the effects of immunostimulation. Tohoku J. Vet. Clin., *21*, 61–70.

Sato S., Suzuki T., Okada K. (1995): Suppression of lymphocyte blastogenesis in cows with puerperal metritis and mastitis. J. Vet. Med. Sci., *57*, 373–375.

Shafer-Weaver K.A., Sordillo L.M. (1997): Bovine CD8+ suppressor lymphocytes alter immune responsiveness during the postpartum period. Vet. Immunol. Immunopathol., *56*, 53–64.

Shafer-Weaver K.A., Corl C.M., Sordillo L.M. (1999): Shiffts in bovine CD4+ subpopulations increase T-helper-2 compared with T-helper-1 effector cells during the postpartum period. J. Dairy Sci., *82*, 1696–1706.

Studder E., Morrow D.A. (1980): Examination and interpretation of findings of the post partum reproductive tract in dairy cattle. From: Morrow D.A.: Current Therapy in Theriogenology, W.B. Saunders, Philadelphia. 287–292.

Van Kampen C., Mallard B.M. (1997): Effects of peripartum stress and health on circulating bovine lymphocyte subsets. Vet. Immunol. Immunopathol., *59*, 79–91.

Corresponding Author

Prof. MVDr. Mikuláš Levkut, PhD., University of Veterinary Medicine, Komenského 73, 041 81 Košice, Slovak Republic
Tel. +421 55 633 81 91, fax +421 55 632 36 66, e-mail: levkut@uvm.sk